Reporter Gene Assay for Fish-Killing Activity Produced by *Pfiesteria piscicida*

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Collaborative studies were performed to develop a functional assay for fish-killing activity produced by *Pfiesteria piscicida*. Eight cell lines were used to screen organic fractions and residual water fraction by using a 3-[4,5-dimethylthiazol-(2-4)]-diphenyltetrazolium bromide cytotoxicity assay. Diethyl ether and a residual water fraction were cytotoxic to several cell lines including rat pituitary (GH_4C_1) cells. Residual water as well as preextracted culture water containing *P. piscicida* cells induced c-*fos*-luciferase expressed in GH_4C_1 cells with a rapid time course of induction and sensitive detection. The reporter gene assay detected activity in toxic isolates of *P. piscicida* from several North Carolina estuaries in 1997 and 1998 and may also be suitable for detecting toxic activity in human and animal serum. *Key words*: assay, c-*fos*, GH_4C_1, *Pfiesteria piscicida*, pituitary, toxin. *Environ Health Perspect* 107:711–714 (1999). [Online 28 July 1999] *http://ehpnet1.niehs.nih.gov/docs/1999/107p711-714fairey/abstract.html*

Pfiesteria piscicida is a toxic dinoflagellate that has been a causative agent of fish epizootics and mortalities in estuaries of the Albemarle–Pamlico Estuarine Ecosystem in North Carolina and the Chesapeake Bay in Maryland (*1*). The possibility for human toxicity became clinically apparent when laboratory scientists working with the toxic algae became ill (*2*). The occurrence of *P. piscicida* in association with fish kills in the Chesapeake Bay led to intensified concern by the state of Maryland and the U.S. Congress. Case–control epidemiologic studies in Maryland demonstrated a significant relationship between contact with water containing *P. piscicida* (*3*) and symptoms that were comparable to those identified in the North Carolina laboratory scientists (*2*), as well as symptoms in rats that were experimentally exposed to the toxic cells (*4*). Efforts to identify the putative toxin have been hampered, in part, by the need for an *in vitro* assay to complement the existing fish toxicity test. Likewise, definitive confirmation of exposure in the Maryland epidemiologic study has been hampered by the lack of a detection method for the putative toxins in humans.

In vitro methods for detecting algal-derived toxins have relied largely on functional assays including receptor-based assays and cell-based toxicity assays (*5,6*). Cell-based assays do not require prior knowledge of the initial cellular target for the toxin, and were chosen as an initial method to screen for possible toxin activity. Selectivity for cell-based assays is based on the varying responsiveness of each cell type because of the combination of receptors, ion channels, enzymes, gene response elements, and other signal-generating molecules unique to that cell. The use of a diverse group of cell types increases the likelihood of identifying an unknown activity.

Cell-based assays can be further modified by changing the end point from the mitochondrial indicator for toxicity {MTT; 3-[4,5-dimethylthiazol-(2-4)]-diphenyltetrazolium bromide (dye-based assay)} to specific gene induction (*7*). These assays, known as reporter gene assays, use responsive cell lines that stably express reporter gene constructs. The constructs should contain the regulatory region of a toxin-inducible gene ligated to the coding region of a reporter element, such as firefly luciferase. The immediate response gene, c-*fos*, has been used as a biomarker of effect for several classes of algal-derived toxins in whole-animal models (*8*). The neuronal cell types expressing c-*fos* differ between toxins, indicating that the cell type conveys some degree of selectivity (*9,10*).

Materials and Methods

Actively growing cultures of *P. piscicida* (Pritchard Pond isolate, Carteret County, NC; identified by H. Glasgow and J. Burkholder through plate suture swelling procedures, with cross-corroboration by K. Steidinger, Florida Department of Environmental Protection, St. Petersburg, FL; unialgal but not axenic) were used for toxin isolation. A second source of material consisted of *P. piscicida* isolate from Beaufort Point (Pamlico County, NC). Toxic cultures were *Pfiesteria* isolates that were maintained in 15 ppt Instant Ocean (Aquarium Systems, Inc., Mentor, OH) in distilled water or sterile filtered seawater (culture water) containing three tilapia (*Oreochromica mossambica*) per tank. Both toxic cultures were taken from tanks that were actively killing tilapia at the time of collection. Whole cultures (50 mL; cells and medium) were partitioned between methylene chloride, diethyl ether, and water, with the water volume reduced via lyophilization. Initially each fraction was reduced to dryness, then brought up in 2 mL of methanol. Using a different type of fish bioassay, all fractions were screened for fish toxicity. This bioassay used sheephead minnows, *Cypronodon variegatus*, that were 7–10 days old. Twenty-four well plates were filled with 1 mL 25 ppt Instant Ocean with one fish per well. The fish were then treated with fractions and observed for 2 hr for toxic effects. The fractions were next screened for cytotoxicity and reporter gene activity assays using the eight cell lines maintained as described below.

Stock cultures of rat pituitary (GH_4C_1) cells were maintained in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (FBS). Stably transfected GH_4C_1 (GH_4C_1-A1) cells were maintained in the same medium as GH_4C_1 cells, but were supplemented with 200 µg/mL neomycin antibiotic (G418; Gibco Life Technologies, Grand Island, NY). Human teratocarcinoma (NT2) cells and mouse hypothalamic (GT1-7) cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS. Mouse neuroblastoma (Neuro2A) cells were maintained in minimum essential medium (MEM) supplemented with 15% FBS. Neuro2A, c-*fos*-luc transfected, cells (N2AC) were maintained in the same medium as Neuro2A cells, but were also supplemented with G418. Human hepatoma (HepG2) cells were maintained in MEM with 10% FBS supplemented with 1 mM sodium pyruvate. All mammalian cultures were incubated at

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37°C with 5% CO_2 and 95% air. Fish cell lines—rainbow trout (RTH-149) cells; Chinook salmon embryo (CHSE-214) cells, and rainbow trout gonadal interstitial (RTG-2) cells—were maintained in MEM supplemented with 10% FBS at 20°C with 5% CO_2 and 95% air. The GH_4C_1 and Neuro2A stable transfectants were obtained by cotransfecting plasmids, c-*fos*-luc and pSV2-neo or pLuc-Link TK-105 and pSV2-neo (Richard N. Day, University of Virginia, Charlottesville, VA), by the calcium phosphate method as previously described (*7*) and were maintained in the above growth medium supplemented with 200 µg/mL G418.

Cytotoxicity was measured using the method of Mosmann (*11*). Each cell line was plated at a density of 30,000 cells/well in 0.1 mL appropriate medium in 96-well tissue culture plates. Serial dilutions of *P. piscicida* extracts and water controls were added to each well and incubated for approximately 24 hr. After incubation, 15 µL MTT [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well and incubated for 4 hr at 37°C. Mitochondrial dehydrogenases in live cells converted the MTT to an insoluble purple formazan crystal. The crystals were solubilized by the addition of 1% sodium dodecyl sulfate (SDS) in 0.1 N HCl and absorbance was read at 570 nm with a Titre-Tek 96-well plate reader (Flow Laboratories, McLean, VA). Nonspecific absorbance by media and nonconverted MTT were subtracted to yield a corrected absorbance value.

GH_4C_1-A1 c-*fos*-luc cells and GH_4C_1-TK-luc cells were seeded in a 96-well clear-bottom white plate (Corning Costar, Cambridge, MA) at a density of 30,000 cells/well in 100 µL cell culture media, and allowed to incubate overnight to ensure attachment. Cells were treated with serial dilutions of *P. piscicida* whole-cell or extracts along with water controls for 4 hr at 37°C. Experimental medium was then removed from wells and 20 µL cell lysis buffer (*7*) was added. Lysis proceeded at room temperature for 20 min, then the plate was placed in a luminometer (RS Luminoskan, Labsystems, Helsinki, Finland). Within the luminometer, each well was injected with 20 µL Luciferase Assay Reagent (Promega, Madison, WI), and the luminescence generated from each well measured over a 10-sec interval.

For the N2AC c-*fos*-luc directed assay, cells were plated at the same density as the GH_4C_1-A1 c-*fos*-luc cells in a 96-well plate. N2AC cells were plated out in serum-free DMEM and allowed to incubate overnight (*7*). Cells were first treated with 0.5 µL veratridine then with serial dilutions of *P. piscicida* fractions and water controls for 12 hr at 37°C. Lysis and plate reading proceeded in the same order as the GH_4C1-A1 c-*fos*-luc assay.

Results

Culture water, organic fractions, and a residual water fraction from toxic cultures of *P. piscicida* were screened for cytotoxicity against a panel of eight cell lines including three neuronal-derived cell lines: Neuro 2A, GT1-7, and NT2 cells; three finfish-derived cell lines: RTH-149, RTG-2, and CHSE-214 cells; and two epithelial cells: HepG2 and GH_4C_1. The cytotoxicity assay end point was the colored product of MTT (*11*), an indicator for mitochondrial function. Culture water containing live *Pfiesteria* cells did not induce cytotoxicity in any of the cell lines (Table 1). The diethyl ether fraction from *P. piscicida* induced cytotoxicity in the Neuro2A, NT2, GH_4C_1, and all three finfish cell lines (RTH-149, RTG-2, and CHSE-214). The residual water fraction induced toxicity in the Neuro2A, HepG2, and GH_4C_1 cell lines. Overall, the GH_4C_1 cells exhibited the highest degree of cytotoxicity from both the diethyl ether and residual water fractions. When the residual water fraction was screened for fish toxicity, however, it showed the highest degree of fish toxicity and killed test fish within 20 min.

We next examined the same fractions on GH_4C_1-A1 and Neuro2AC cells stably expressing the c-*fos*-luciferase reporter gene. Toxic culture water containing live *Pfiesteria* cells and residual water fractions, each given at the highest noncytotoxic concentrations (determined in half-log concentration orders) induced reporter gene activity in the GH_4C_1-A1 cells, but not in the N2AC cells (Table 2). No activity was found for the diethyl ether fraction in either cell line. Later, we examined GH_4C_1 cells transfected with a low-level promoter ligated to the luciferase reporter, TK-luc reporter gene. Decreasing concentrations of extracted *P. piscicida* were added to each well. No activity was found with this reporter gene system. Concentrated salts induced c-*fos* luciferase in GH_4C_1-A1 cells. When concentrated Instant Ocean Salt Solution (Aquarium Systems, Inc.) was applied to the assay, concentrations of ≥ 5-fold of the 15 ppt mixture induced c-*fos* luciferase. Analysis of the individual components of the 5-times concentrated mixture indicated that the active component was sodium (*12*). Hence, concentrates of the culture water could not be used for the assay without the removal of salts.

A time course of luciferase induction was completed for the residual water fraction. Induction nearly doubled after 2 hr and increased further between 2 and 4 hr and remained at high levels of induction at 8 hr (Figure 1). Thus, a treatment time of 4 hr was chosen for this assay. Sensitivity of the assay was determined by adding decreasing concentrations of the toxic culture water containing living *P. piscicida* cells and residual water after extraction to GH_4C_1-A1 cells. Both preparations gave parallel concentration curves with an increase occurring over two log orders of concentration (Figure 2). The concentration dependency was related to cell number based on counts of the number of toxic organisms present in the water. The detection range was approximately 3–30 cells/well. At relative cell concentrations of 30 cells/well the residual water fraction and

Table 1. Cytotoxicity of *Pfiesteria piscicida* culture fractions on eight cell lines.

Cell line	Water control	Pritchard Pond culture water[a]	Methylene chloride	Diethyl ether	Residual water
Neuro2A	1.130	1.015	1.020	0.642*	0.902*
GT1-7	0.539	0.368	0.312	0.412	–
NT2	0.826	1.002	0.169*	0.680*	0.821
RTH-149	0.413	0.465	0.429	0.286*	0.487
RTG-2	0.410	0.546	0.420	0.266*	0.460
CHSE-214	0.457	0.550	0.464	0.256*	0.548
HepG2	0.493	0.504	0.434	0.406	0.304*
GH_4C_1	0.619	0.523	0.606	0.297*	0.266*

Abbreviations: CHSE-214, Chinook salmon embryo; GH_4C_1, rat pituitary; GT1-7, mouse hypothalamic; HepG2, human hepatoma; Neuro2A, mouse neuroblastoma; NT2, human hepatocarcinoma; RTG-2, rainbow trout gonadal interstitial; RTH-149, rainbow trout.
[a]Preextracted (*P. piscicida* live cells). The data represent the mean of duplicate wells with absorbance readings at 570 nm. *Significantly different from controls [Tukey multiple comparison test (*17*); $p < 0.05$].

Table 2. Reporter gene assay activity of *Pfiesteria piscicida* culture fractions on two stably transfected cell lines.[a]

Cell line	Water control	Pritchard Pond culture water[b]	Methlyene chloride	Diethyl ether	Residual water
GH_4C_1	144	304.5*	ND	159.7	351.5*
Neuro2AC	72.6	62.2	ND	56.1	81.8

Abbreviations: GH_4C_1, rat pituitary; ND, not detectable. Neuro2AC, mouse neuroblastoma.
[a]Volume of sample with the highest no effect level for the corresponding cytotoxicity assay. [b]Preextracted (*P. piscicida* live cells). The data represent the mean of duplicate wells reported in relative light units. *Significantly different from controls [Tukey multiple comparison test (*17*); $p < 0.05$].

culture water inhibited luciferase activity and at 100 cells/well both were cytotoxic on the MTT assay. The concentration dependency developed a bell-shaped curve. The reporter gene assay has also been used to detect activity in four North Carolina *P. piscicida* cultures from Pritchard Pond and Beaufort Point isolates of 1997 and Vanderemere and Beaufort I isolates of 1998.

Selectivity of the reporter gene assays to other algal toxins such as brevetoxin-1 (PbTx-1), ciguatoxin (CTX-C3), saxitoxin (STX), and domoic acid (DA) was determined. None of these algal-derived toxins induced the reporter gene in GH_4C_1-A1 cells; however, PbTx-1 and CTX-C3 induced reporter gene activity, and STX inhibited activity in Neuro2AC cells (Figure 3).

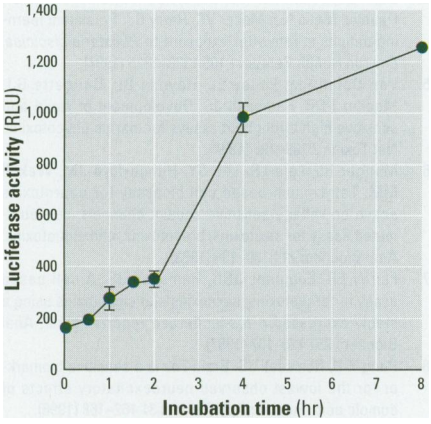

Figure 1. Time course for induction of c-*fos* luciferase. RLU, relative light units. Rat pituitary (GH_4C_1-A1) c-*fos*-luc cells were treated with 10 µL residual water after extraction from the Pritchard Pond isolate of *Pfiesteria piscicida*. At times ranging between 0 and 8 hr, cells were lysed and the activity of the solubilized luciferase protein was measured in a microplate luminometer. Each point represents the mean ± standard error of four wells for a representative experiment, repeated once with similar results.

The effect of serum as a matrix for the reporter gene assay was determined by spiking human serum with the residual water fraction of *P. piscicida*. A given amount of the residual water fraction was combined with increasing volumes of human serum and this mixture was then added to the assay. The assay detected activity when the residual water fraction was mixed with as much as 20 µL human serum (Figure 4). In a separate set of experiments, stability of the residual water fraction was determined in human serum. Human serum was spiked and aliquots were frozen at -80°C or lyophilized and compared to lyophilized residual water and retested following 1-, 8-, 30-, and 90-day storage at -80°C. No decrease in percent induction of c-*fos* luciferase was found and values for 10 µL of the -80°C frozen serum, lyophilized serum, and lyophilized residual water were within 20% for each of the times tested (*12*).

Discussion

The goal of this study was to provide tools necessary to enhance research on *P. piscicida* and its toxins. Specifically, we sought to develop a functional assay to guide purification of the toxin(s) and to provide a means to screen for toxic activity in algal cultures and perhaps biological fluids. Toxic activity was assessed using a panel of cytotoxicity assays, which identified activity in both lipid-soluble and water-soluble components. The GH_4C_1 cells showed the largest cytotoxic response to both the diethyl ether and residual water fractions. However, only the residual water fraction induced the reporter gene activity in these cells. Taken together, these results may suggest that more than one form of the putative toxin exists. Because the reporter gene assay requires approximately 100 times less active material than the fish assay, it is the most practical means to guide fractionation of the toxin. Purification of the water-soluble activity by several chromatographic procedures shows that fractions containing the reporter gene activity coelute with fish-killing activity (*13*), indicating that the toxic activity of these fractions may be responsible for the unusual fish mortality events associated with *P. piscicida*.

This report describes the initial characterization of a functional assay for fish-killing toxins produced by *P. piscicida*. The reporter gene assay in GH_4C_1-A1 cells shows some degree of selectivity for algal-derived toxins. PbTx-1, CTX-C3, STX, and DA do not induce c-*fos*-luciferase in the GH_4C_1-A1 cells. In Neuro2AC cells, PbTx-1 and CtX-C3 induce and STX inhibits PbTx-1-induced c-*fos*-luciferase. This degree of selectivity is due to the higher complement of voltage-dependent sodium channels in the Neuro2A cells. It is likely that other agents induce c-*fos*-luciferase in GH_4C_1-A1 cells. Five-times concentrated seawater or Instant Ocean Sea Salts induce the c-*fos*-luciferase

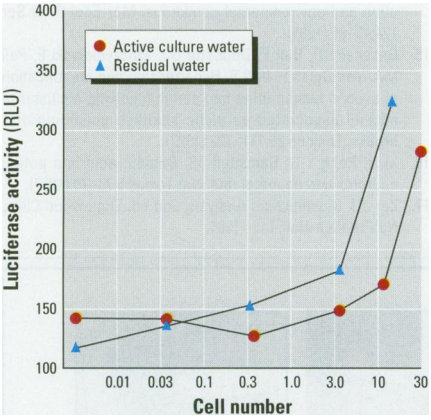

Figure 2. Concentration dependency for induction of c-*fos* luciferase. RLU, relative light units. Stably transfected rat pituitary (GH_4C_1-A1) c-*fos*-luc cells were treated with increasing concentrations of preextracted culture water or residual water after extraction from the Pritchard Pond isolate of *Pfiesteria piscicida*. At 8 hr, cells were lysed and the activity of the solubilized luciferase protein was measured in a microplate luminometer. Each point represents the mean of two wells for a representative experiment repeated once with similar results.

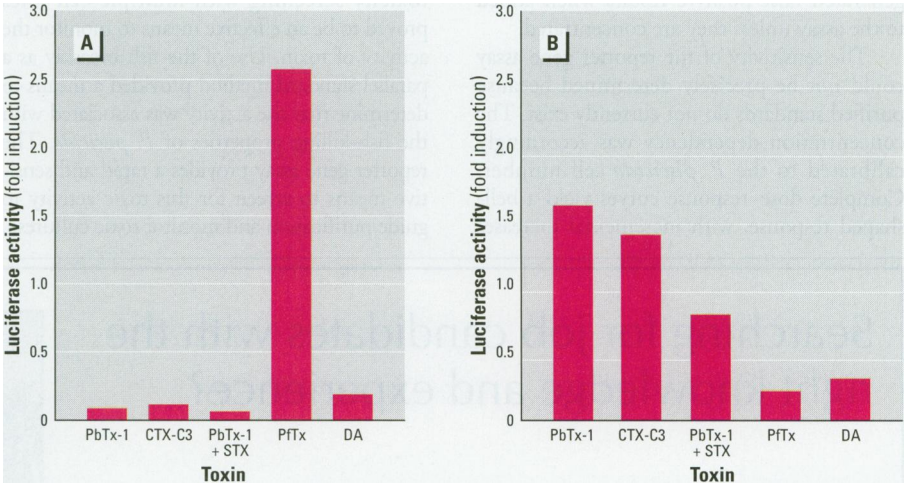

Figure 3. Selectivity for induction of c-*fos* luciferase in (*A*) GH_4C_1 c-*fos* and (*B*) Neuro2A c-*fos* cells. Abbreviations: CTX-3C, ciguatoxin; DA, domoic acid; GH_4C_1-A1, rat pituitary; Neuro2AC, mouse neuroblastoma; PbTx-1, brevetoxin-1; PfTx, *Pfiesteria piscicida*; STX, saxitoxin. GH_4C_1-A1 c-*fos*-luc cells and Neuro2AC c-*fos*-luc cells were treated with the following algal-derived toxins: PbTx-1 (10 ng/mL); CTX-3C (3 pg/mL); STX (10 nM) plus PbTx-1; residual water after extraction from the Pritchard Pond isolate of PfTx (10 µL); or DA (10 nM). GH_4C_1-A1 cells were lysed at 4 hr and Neuro2AC cells at 12 hr, and the activity of the solubilized luciferase protein was measured in a microplate luminometer. Each bar represents the fold induction of the reporter gene over vehicle for a representative experiment, repeated once with similar results.

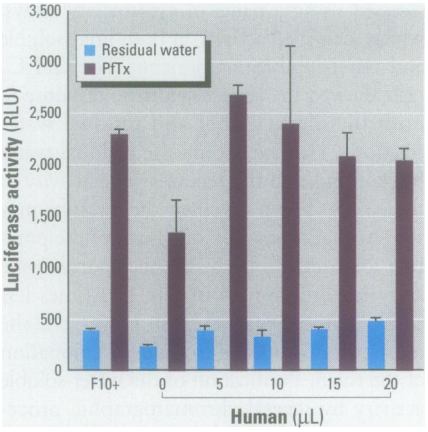

Figure 4. Detection of residual water fraction activity in spiked serum. Abbreviations: GH_4C_1-A1, stably transfected rat pituitary; PfTx, *Pfiesteria piscicida;* RLU, relative light units. GH_4C_1-A1 c-*fos*-luc cells were treated with water or the residual water after extraction from the Pritchard Pond isolate of PfTx (10 µL) that had been preequilibrated with increasing volumes of human serum. GH_4C_1-A1 cells were lysed at 4 hr and the activity of the solubilized luciferase protein was measured in a microplate luminometer. Each bar represents the mean ± standard error of 4 wells for a representative experiment, which has been repeated with serum from rat, dolphin, and menhaden.

reporter gene, but no effects were seen with nonconcentrated water. The application of appropriate Instant Ocean concentrations controls for seawater effects. If salts are concentrated, they must be removed. A method for salt removal still must be developed. However, biological fluids, such as human serum, or media, such as seawater or aquarium water, conditioned with fish have not generated false-positive results when added to the assay unless they are concentrated.

The sensitivity of the reporter gene assay could not be precisely determined because purified standards do not currently exist. The concentration dependency was accordingly calibrated to the *P. piscicida* cell number. Complete dose–response curves yield a bell-shaped response, with incremental increases in luciferase activity until inhibition of luciferase activity presumedly due to cytotoxicity co-occurs; this in turns leads to decreases in luciferase. Culture water containing 100 cells/well (0.1 mL) of the Pritchard Pond isolate of *P. piscicida* inhibits reporter gene activity and 30 cells causes a maximal induction of luciferase. When the residual water fraction extracted from this culture is tested, a 30-cell equivalent is inhibitory and a 10-cell equivalent causes a maximal induction. One potential explanation why the residual water fraction is more potent that the culture water is because the cells have been lysed and their contents released.

Because the density of toxic cells ranges from approximately 250–250,000 cells/mL in the wild during fish kills (*2,14*), the detection limit of the assay may be suitable for testing unprocessed water samples. We anticipate that the assay may also be suitable for measuring the presence of the activity in exposed animals, such as fish at the site of a mortality event, or perhaps humans. Water-soluble algal toxins such as STXs and DA have been measured in the serum of acutely exposed humans and laboratory animals by receptor assays (*15,16*). In the only published laboratory animal study of *Pfiesteria* toxins to date (*4*), injection of 30,000 and 100,000 cells/kg generated neurobehavorial effects in rats. However, the reporter gene assay will only serve as a screen for toxic activity; detection of the putative toxins directly will require chemical analysis.

Current studies are directed at purifying the component(s) causing fish-killing activity and elucidating its structure. The use of cytotoxicity screening with multiple cell lines proved to be an effective means to monitor the activity of toxin. Use of the fish bioassay as a parallel standard method provided a means to determine that the activity was associated with the fish-killing properties of *P. piscicida.* The reporter gene assay provides a rapid and sensitive means to screen for this toxic activity to guide purification and monitor toxic cultures.

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